Effects of cyclic AMP and theophylline on chloride conductance across toad skin

U. Katz and W. Nagel*†

*Department of Biology, Technion, Israel Institute of Technology, Haifa, Israel and *Physiologisches Institut der Universität, Munich, Germany*

1. The effects of the phosphodiesterase inhibitors theophylline and isobutylmethylxanthine (IBMX) on baseline and voltage-activated Cl^- conductance (g_{Cl}) of toad skin were compared with those of the potent 2-chlorophenylthio analogue of cAMP (CPT-cAMP).

2. Using intact and split skins of *Bufo viridis* we confirmed that theophylline and IBMX raised the voltage-activated g_{Cl} with a pattern identical to that seen under control conditions. This effect was small or missing if g_{Cl} was already high in the control.

3. CPT-cAMP, in contrast, increased the Cl^--specific conductance by up to 6 mS cm^{-2} at short circuit. The characteristic time-dependent, slow activation of g_{Cl} by serosa-positive clamp potentials was completely lost under these conditions.

4. Coinciding with the loss of voltage activation of g_{Cl} the plateau value of the Lorentzian component of fluctuation in current at serosa-positive clamp potentials decreased by almost 50%. The corner frequencies were not notably different.

5. After CPT-cAMP, the sigmoidal voltage–conductance relation that is characteristic of control conditions or after theophylline disappeared; the patterns were variable and incompatible with voltage activation.

6. The voltage-activated g_{Cl} under control conditions and with theophylline was blocked by mucosal NO_3^-, I^- or SCN^-, the last two being almost equally effective. In the presence of CPT-cAMP, mucosal NO_3^- had minimal influence on tissue conductance, whereas the effects of I^- and SCN^- were essentially unchanged. Br^- on the mucosal side could substitute for Cl^- under all conditions.

7. The results suggest that protein phosphorylation by supramaximal concentrations of cAMP induces maximal conductance through anion-specific routes, while the voltage sensitivity of this pathway is lost. The effects of theophylline and IBMX on the voltage-activated Cl^- conductance of toad skin cannot be explained solely by inhibition of the phosphodiesterase.

Transepithelial Cl^- conductance of the amphibian skin is affected by phosphodiesterase inhibitors such as theophylline as well as by cAMP (Cuthbert & Painter, 1968; Katz & Van Driessche, 1988) and it was supposed from electrophysiological data that the effect involved cAMP-mediated regulation of the Cl^--specific, voltage-activated pathway (Willumsen, Vestergaard & Larsen, 1992). This pathway, which accounts for a large fraction of Cl^- transport across the toad and frog skin, can be activated by serosa-positive electrical potential differences. Cl^- movement occurs passively and is driven by the electrochemical gradient. As a morphological site for transport, mitochondria-rich (MR) cells were proposed in toad and frog skin (see Larsen, 1991; Katz & Nagel, 1994, for reviews). A Cl^--specific conductance is also observed under short-circuit conditions. It appears that this occurs more frequently in the skin of frogs than those of toads (Kristensen, 1983; Nagel, Natochin & Crabbe, 1988*b*). Furthermore, Cl^- can cross the epithelium via unspecific conductive leak pathways; these pathways are often considered rather tight, but conditions may exist with elevated permeability (Watlington & Jessee, 1973).

The relation between Cl^- conductance at short circuit and the voltage-activated chloride conductance (g_{Cl}), and their respective modulation by cAMP are not yet clear, and we have therefore investigated the effect of two inhibitors of the phosphodiesterase (theophylline and isobutylmethylxanthine (IBMX)), as well as that of forskolin and two

† To whom correspondence should be addressed.

analogues of cAMP on the Cl^- conductance across toad skin. All these substances are believed to increase the concentration of cellular cAMP (which serves as a second messenger), affecting the biological process involved through protein phosphorylation (Berridge, 1986). Our findings show that these substances can be divided into two groups according to their effects on the Cl^- conductance in the toad skin. Cyclic AMP and forskolin increase the g_{Cl} considerably, but remove its sensitivity to voltage activation, while theophylline and IBMX increase the baseline conductance a little, and sensitize the voltage activation of Cl^- conductance.

METHODS

Toads (*Bufo viridis*) of both sexes were used. They were kept at the laboratory with free access to tap water and were fed meal worms every second week. The animals were killed by double pithing. Abdominal skins were carefully dissected and used either intact (whole skin) or after removing the dermis (split skin) following a 1·5–2 h incubation in Ringer solution containing 1 mg ml^{-1} collagenase (Type II, Sigma). The tissues were mounted in a modified Ussing-type chamber (Nagel & Van Driessche, 1992) and were continuously perfused on both sides. The serosal perfusion solution was always aerated Ringer solution of the following composition (mM): Na^+, 115; K^+, 2·5; Ca^{2+}, 1; Cl^-, 117; HCO_3^-, 2·5; pH 8·0. The mucosal solution had the same ionic composition except that HCO_3^- was replaced by 3·5 mM Hepes, which resulted in a pH of 7·6. Changes in the composition of this Ringer solution were made by equimolar replacements of Na^+ or Cl^-. Na^+ transport was eliminated by substitution of *N*-methyl-D-glucamine (NMDG) for Na^+ when the fluctuation in current noise was analysed. In most other experiments, amiloride (10^{-5} M) was used to block Na^+ transport.

The preparations were maintained at short circuit or voltage clamped to +30 mV (with reference to the serosal side) to ensure deactivation of the voltage-sensitive Cl^--specific conductance, g_{Cl} (Larsen & Kristensen, 1976). Transepithelial current (I_t) and small pulse tissue conductance (g_t) were determined either using a custom-made voltage clamp device with sample-and-hold circuitry (Nagel, Garcia-Diaz & Essig, 1983) or with a low-noise voltage clamp device (Van Driessche & Gullentops, 1982) connected via a CIO-AD16 12-bit AD/DA-interface (Computer Boards Inc., Mansfield, MA, USA) to a PC-class computer. In the latter case, g_t was continuously estimated from the change in I_t on perturbations of V_t by 3–5 mV for 256 ms; software routines for data sampling and computation were kindly provided by Professor W. Van Driessche, KULeuven, Belgium. Records of I_t and g_t *versus* time were produced from these files using standard graphics software (Origin, MicroCal Software Inc., Northampton, MA, USA). Transepithelial potential difference (V_t) is referred to the serosal side of the tissue. It is considered negative for anion flux from mucosa to serosa. Clamp potentials of −80 mV were chosen for the analysis of the voltage-activated Cl^- pathway, since g_{Cl} becomes maximally activated at these values (Larsen & Rasmussen, 1983).

Equipment used for recording and analysing the power density spectra (PDS) of the fluctuation in current has been described previously (Van Driessche & Gullentops, 1982; Van Driessche & Erlij, 1988). As discussed in previous publications (see e.g. Van Driessche & Zeiske, 1985), the presence of a Lorentzian component in the noise spectrum is associated with random interruptions of current through gated ion channels. In this sense, the occurrence of Lorentzians is considered to indicate existence of fluctuating anion channels. All drugs were purchased from Sigma. Experiments were performed at room temperature (23–27 °C). Where appropriate, mean values are reported ± S.E.M.; significance of difference was calculated using Student's t test considering $2P < 0{\cdot}05$ as significant.

RESULTS

Transepithelial Cl^- current and conductance

More than thirty preparations of both intact skins and split epithelia from fourteen animals were used in this study. Transport activity measured as short-circuit current (I_{sc}) in identical NaCl Ringer solution on both sides was 20–70 $\mu A\ cm^{-2}$ at the beginning of the experiments and not systematically different between intact skins and split epithelia. After addition of amiloride (10^{-5} M) or replacement of mucosal Na^+ by NMDG, I_{sc} decreased consistently to values close to zero; reversed I_{sc} that would indicate proton secretion was never observed. At this time (approximately 15–25 min after the mounting of the skins), g_t at short circuit was variable, ranging from 0·3 to 2·1 mS cm^{-2}. If g_t was initially high, it decreased (always spontaneously) during equilibration, reaching values below 1 mS cm^{-2} within some 20–30 min. After replacement of apical Cl^- with SO_4^{2-}, g_t decreased to less than 0·3 mS cm^{-2}. This indicates that elevated conductance was not caused by edge damage effects but was due to a Cl^--specific component. Dissipation of elevated conductance was in most experiments enhanced by voltage clamping of the tissue to +30 mV (Larsen & Kristensen, 1976) and the skins were kept at this potential between voltage perturbations. For activation of Cl^--specific conductive pathways, tissues were temporarily hyperpolarized to values of −80 mV and maintained in this state until a new steady state level of g_t was approached (usually less than 4 min). After several measurements under control conditions, activators of the Cl^--conductive pathway were applied and intermittent voltage perturbations were continued.

Figure 1*A* shows the response pattern of g_t in a split skin analysed in the presence of Na^+ transport. Baseline conductance (at short circuit) was 0·3 mS cm^{-2} and decreased slightly after clamping V_t to −80 mV. Such a response, which is presumably due to decrease in apical membrane Na^+ conductance (Nagel, Garcia-Diaz & Essig, 1988*a*) associated with minimal increase in voltage-activated g_{Cl}, was occasionally observed although it is uncharacteristic for skins from *Bufo*. (Frog skin is often lacking responsiveness of g_{Cl} to voltage activation under control conditions and its sensitization by agents such as theophylline or procaine is also unpredictable; Nagel, 1989; Nagel & Dörge, 1990.) Mucosal addition of 1 mM theophylline increased the baseline (short-circuited) g_t very slightly, but clearly increased the voltage sensitivity of the Cl^- pathway; g_t stabilized at about 1·0 mS cm^{-2} within

2 min after clamping V_t to −80 mV. Subsequent addition of 100 μM CPT-cAMP (2-chlorophenylthio analogue of cAMP) to the serosal solution increased the baseline conductance from 0·4 to more than 2·0 mS cm^{-2}. Early under these conditions, the sensitivity of g_t to voltage activation was greatly reduced. A second voltage perturbation to −80 mV, done after approach of the steady state during CPT-cAMP, increased g_t only slightly and transiently. Replacement of apical Cl^- by SO_4^{2-} decreased g_t to values only slightly above the initial baseline level. This shows that the major fraction of the cAMP-induced conductance was Cl^- specific. In Fig. 1*B* we show another experiment with a similar protocol. Here, voltage activation of g_t was already substantial under control conditions. Mucosal theophylline increased the baseline conductance at short circuit clearly, but the gain in g_t after voltage clamping to −80 mV was smaller. Thus, the combined effect of theophylline and voltage activation under control conditions increased g_t only slightly more than voltage clamping alone. CPT-cAMP, on the other hand, notably increased baseline g_t as observed in the above experiment, and completely eliminated the sensitivity of g_t to voltage activation. Washout of theophylline and CPT-cAMP returned g_t rapidly back to the low level observed in the control state; voltage sensitivity of g_t was then also recovered.

The maximal response to voltage perturbation, phosphodiesterase inhibitors and cAMP-analogues was always clearly reduced in split epithelium compared with the intact skin. Voltage-activated conductance in split skins with or without theophylline and g_t after CPT-cAMP were, respectively, 53% ($n = 5$), 46% ($n = 7$) and 47% ($n = 5$) of the values in intact skins from the same animals. Response patterns, however, were identical in these groups. The response of split skins was not influenced by the number of glands remaining after the splitting procedure. This was verified in two experiments, in which several skin pieces of the same animal were incubated with collagenase for varying times between 60 and 150 min in order to alter the density of remaining intact glands. Using this technique, between 0 and 70% of glandular ducts were associated with morphologically intact glands. The increase in g_t was similar in all pieces and unrelated to the density of the

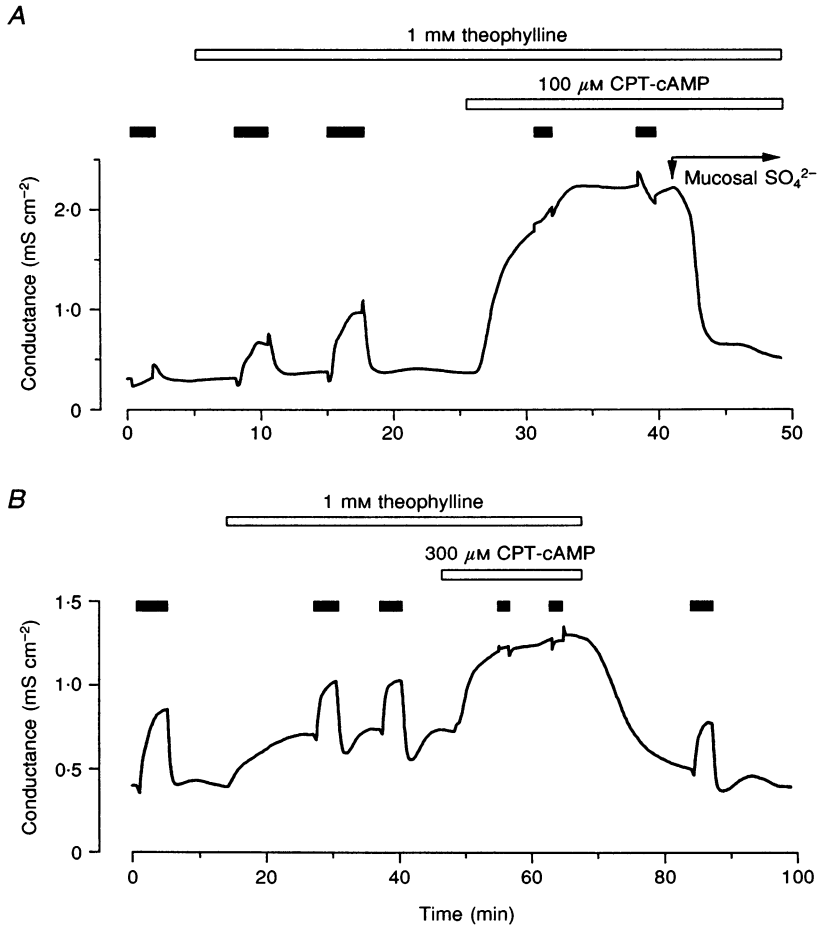

Figure 1. Time course of transepithelial conductance (g_t) of isolated toad skin

The tissues were short circuited except for periods indicated by the filled boxes, during which V_t was clamped to mucosa at −80 mV. See text for further explanation. Note, the tissue in *A* was analysed in the presence of Na^+ transport, i.e. with NaCl Ringer solution without amiloride on the apical side.

Table 1. Transepithelial baseline conductance (g_t) at short circuit of intact toad skin and effect of voltage perturbation to $V_t = -80$ mV on g_t under control conditions and after application of 2 mM theophylline or 100–300 μM CPT-cAMP

	g_t (mS cm^{-2})		
	Control ($n = 12$)	Theophylline ($n = 12$)	CPT-cAMP ($n = 11$)
Short circuit	0·70 ± 0·13	0·93 ± 0·19	3·58 ± 0·53
$V_t = -80$ mV	2·30 ± 0·32	3·10 ± 0·38	3·39 ± 0·49

Mean values ± S.E.M.

remaining glands. Thus, effects are essentially due to responses of the epithelium itself.

Table 1 shows that baseline tissue conductance at short circuit increased slightly after theophylline (by some 25%) and almost 5 times after CPT-cAMP. In the presence of theophylline, voltage sensitivity of g_t was significantly larger than under control conditions, and IBMX gave a similar response (data not shown). However, the latter drug was considerably more potent than theophylline in molar terms; 100 μM IBMX elicited maximal stimulation of g_t, compared with more than 2 mM that was required in the case of theophylline. After application of CPT-cAMP, the magnitude of g_t was clearly larger than the voltage-activated g_t under control conditions; it was only slightly and not significantly larger than the voltage-activated g_t with theophylline. Voltage perturbation to −80 mV in the presence of CPT-cAMP decreased g_t notably, which is contrary to the response under control conditions and with theophylline. In four separate experiments, theophylline was added after full activation with CPT-cAMP that was accompanied by complete elimination of the voltage-activated component. This did not further increase the baseline conductance (at short circuit), but a slight time-dependent activation of g_t, associated with voltage perturbation to −80 mV, was thereafter observed in all cases.

Voltage activation of g_{Cl} was repeatedly tested by intermittent hyperpolarization from +30 to −80 mV; changes of I_t reflected g_t, while V_t was maintained at the clamp potential. Results of a typical experiment are shown in Fig. 2, which displays the time course of I_t for control conditions and with three concentrations of CPT-cAMP. In the control, I_t changed immediately after the hyperpolarization as predicted by the magnitude of g_t. Thereafter, approach to the steady-state level with increased g_t ensued after a short delay. The time course of this current change can be described by a single exponential with time constant $\tau = 68$ s. Application of 30 μM CPT-cAMP increased the magnitude of the instantaneous change in I_t slightly and reduced the subsequent response resulting from voltage activation. These effects became more pronounced at 100 and 300 μM CPT-cAMP; at the higher concentration, I_t was lacking any activation by voltage, but rather decreased during the perturbation of V_t. Similar results were obtained in all twelve experiments. The concentration of CPT-cAMP sufficient to remove the voltage sensitivity of current was between 100 and 300 μM, with the lower concentrations requiring longer periods of

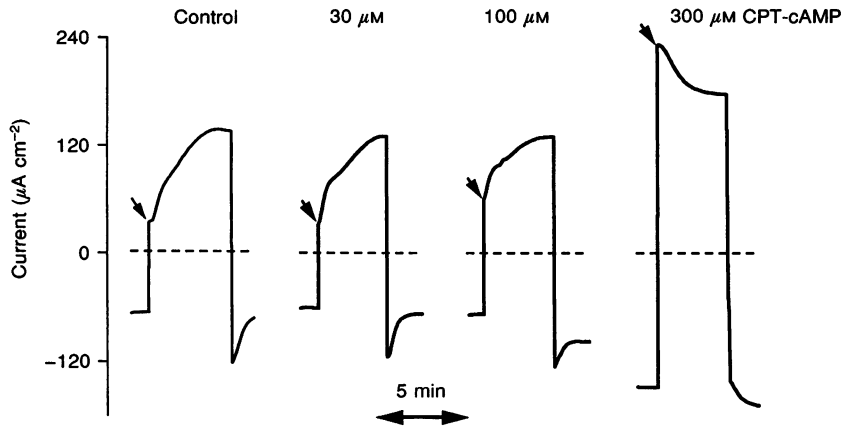

Figure 2. Time course of transepithelial current (I_t) of intact toad skin, voltage clamped to +30 mV with intermittent perturbation to −80 mV

CPT-cAMP was added in increasing concentration to the serosal side approximately 20 min before the depicted sequences. Arrows point to the instantaneous values of current after voltage perturbation.

Table 2. Transepithelial current at $V_t = -80$ mV and plateau value (S_0) of the Lorentzian of fluctuation in current for toad skin under control conditions and after steady-state stimulation with 100–300 μM CPT-cAMP on the serosal side (AMP)

	I_t (μA cm^{-2})			S_0 (10^{-21} A^2 s cm^{-2})		
	Control	AMP	Ratio	Control	AMP	Ratio
	190	183	0·96	29·2	13·5	0·46
	138	178	1·29	28·0	19·0	0·68
	332	356	1·07	77·1	31·4	0·41
	253	304	1·20	44·1	21·1	0·48
	106	99	0·93	30·9	15·1	0·49
	82	235	2·87	61·3	39·0	0·63
	137	151	1·08	89·2	37·0	0·43
	40	88	2·20	24·1	17·1	0·71
	60	106	1·77	17·8	8·2	0·46
Mean	149·0	188·9	1·49	44·6	22·5	0·53
S.E.M.	31·6	31·2	0·22	8·5	3·7	0·04

pre-incubation. Considerably lower potency was observed for dibutyryl-cAMP (dB-cAMP), which increased g_t at a concentration of 500 μM to 86 ± 15% ($n = 4$) of the values observed in matched tissues with 100 or 300 μM CPT-cAMP. Qualitatively, however, the responses of g_t on application of either of the cAMP analogues were similar. Theophylline, in contrast, had a fundamentally different effect on the time course of voltage activation. This is shown in a typical experiment in Fig. 3, where both the baseline conductance and the magnitude of slow voltage activation of I_t were clearly larger after theophylline compared with the control. The time course of the approach to the new steady-state level of I_t was shorter after theophylline than in the control ($\tau = 56$ *vs.* 87 s). Consecutive application of CPT-cAMP completely eliminated the time-dependent, voltage-activated g_t. It is noteworthy that, under the latter conditions, g_t changed immediately and notably upon voltage perturbation. It was not possible to estimate a value for τ after the application of CPT-cAMP, because both current and conductance responded fast, approaching the final level within a few seconds.

Noise measurements

Fluctuation in voltage-activated Cl^- current shows a Lorentzian component in addition to unspecific $1/f$ noise in the lower frequency range and amplifier noise at high frequencies (Nagel & Van Driessche, 1991). The Lorentzian component is characteristically affected by CPT-cAMP. Power density spectra of a typical experiment are shown in Fig. 4. All spectra were recorded at $V_t = -80$ mV, when I_t was 137, 136 and 151 μA cm^{-2} under control conditions and after serosal addition of CPT-cAMP (60 and 300 μM), respectively. The plateau value (S_0) of the Lorentzian decreased from $89{\cdot}2 \times 10^{-21}$ A^2 s cm^{-2} under control conditions to $46{\cdot}4 \times 10^{-21}$ and $37{\cdot}1 \times 10^{-21}$ A^2 s cm^{-2} with 60 and 300 μM CPT-cAMP, respectively. Corner frequencies (f_c) were between 60 and 70 Hz, but were not notably different with CPT-cAMP from the control values. Similar results were obtained in all nine experiments, which are summarized in Table 2. On average, I_t at −80 mV increased significantly ($2P < 0{\cdot}05$) by about 25%, whereas S_0 was reduced to half of the control value ($2P < 0{\cdot}001$). The value of f_c was not significantly different between control and CPT-cAMP (76 ± 13 *versus* 86 ± 19 Hz; $2P > 0{\cdot}2$). The inhibitory effect of CPT-cAMP on S_0 of the Lorentzian was reversible in a similar way to the gain in I_t. As reported previously (Nagel & Van Driessche, 1991), theophylline had no consistent effect on the S_0 of the Lorentzian despite a large increase in voltage-activated current.

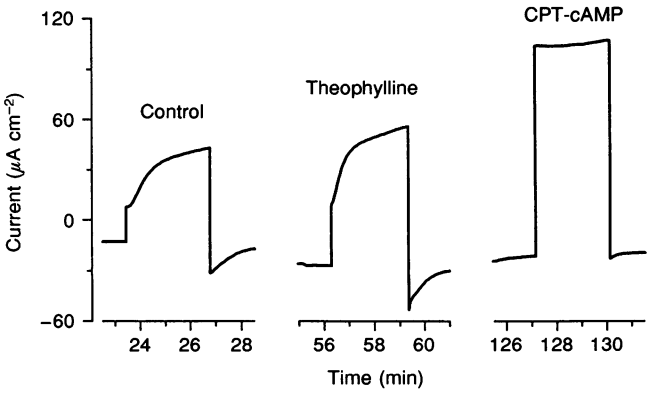

Figure 3. Response of transepithelial current (I_t) on voltage perturbation from +30 to −80 mV

Response of transepithelial current I_t on voltage perturbation from +30 to −80 mV under control conditions, in the presence of 1 mM theophylline in the mucosal perfusion solution and with 300 μM CPT-cAMP in the serosal solution.

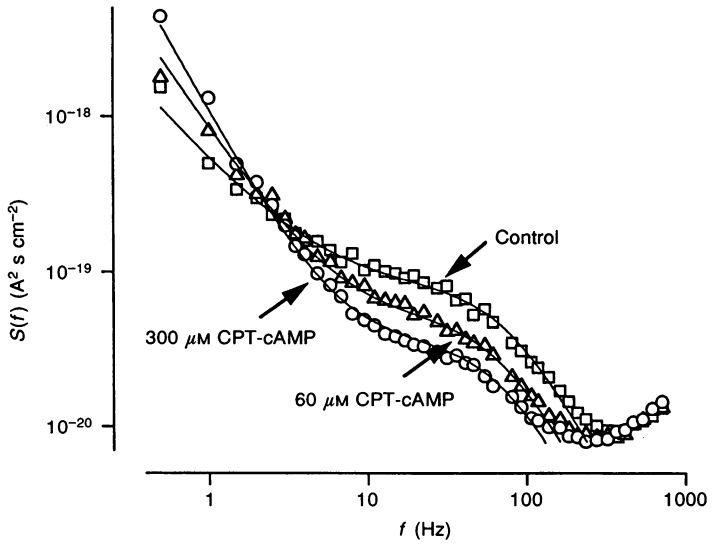

Figure 4. Power density spectra of the fluctuation in voltage-activated Cl^--specific transepithelial current

Power density spectra of the fluctuation in voltage-activated Cl^--specific transepithelial current of toad skin under control conditions and after serosal application of CPT-cAMP in two concentrations. Measurements were made during steady states before and after addition of 60 or 300 μM CPT-cAMP.

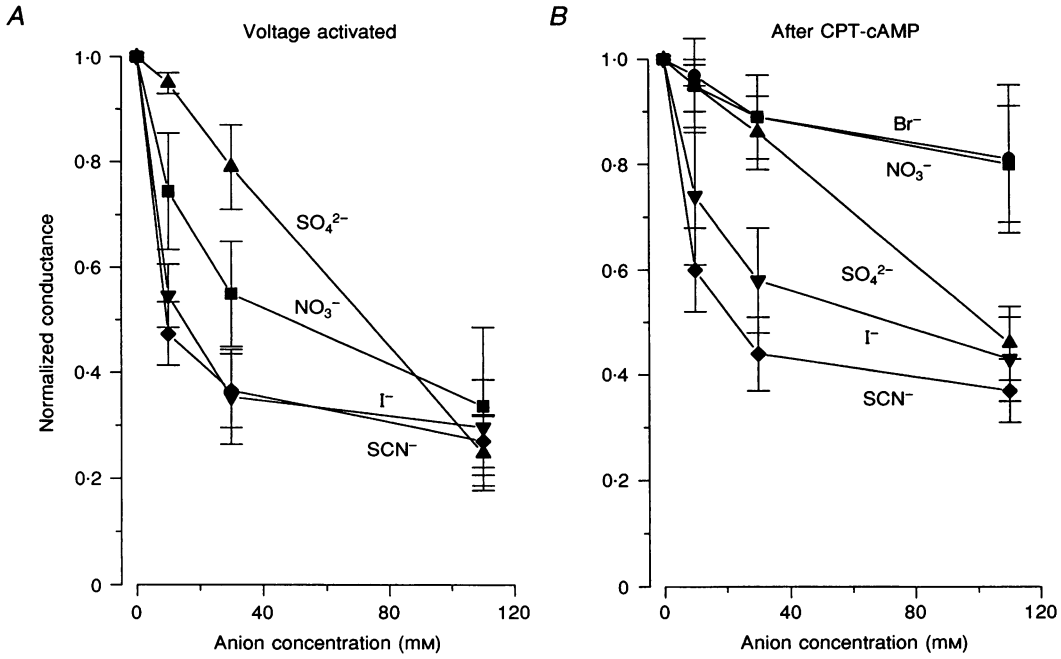

Figure 5. Response of tissue conductance (g_t) of toad skin on equimolar replacement of apical Cl^- by different anions

A, effect on voltage-activated g_{Cl}. The tissues were maintained at −80 mV and substitution was made with increasing concentrations of the respective anions. Before turning to a different anion, the tissues were intermittently returned to NaCl Ringer solution. *B*, response of CPT-cAMP-induced g_t at short circuit. Otherwise as before. Data from 4–7 tissue preparations for each condition.

Selectivity of the Cl^- conductance pathway

The selectivity of the Cl^- conductance pathway changed considerably following incubation with CPT-cAMP. These observations are summarized in Fig. 5. Figure 5*A* shows the influence of a number of anions on the magnitude of the voltage-activated g_{Cl}, and Fig. 5*B* shows the effect of the same anions following application of CPT-cAMP; the latter determinations were done at short circuit, since the cAMP-induced conductance is insensitive to voltage. It is evident that voltage activation of g_{Cl} required the presence of apical Cl^-. SO_4^{2-} decreased g_t essentially as predicted by the reduction of the apical Cl^- concentration. NO_3^-, SCN^- and I^- apparently inhibited g_t, the latter two being almost identical in inhibitory potency and slightly more effective than NO_3^-. After CPT-cAMP, tissue conductance became almost insensitive to NO_3^- and remained comparable to that during the presence of Cl^- in the presence of apical NO_3^- as well as that with Br^- in the apical solution. No difference was apparent in the decrease of g_t after replacement of Cl^- with SO_4^{2-}. Also, SCN^- and I^- similarly inhibited the CPT-cAMP-induced g_t as well as the voltage-activated conductance.

Voltage-related conductance (g–V relations)

The typical dependence of the voltage-sensitive Cl^- conductance on the clamping potential for skins of *Bufo viridis* is shown in Fig. 6 for control conditions and after incubation with 1 mM theophylline (Fig. 6*A*) or after addition of 300 μM CPT-cAMP (Fig. 6*B*). Under control conditions, g_{Cl} was almost completely inactivated at short circuit, decreasing marginally at positive V_t. Hyperpolarizing V_t caused the usual increase in g_t. Theophylline, which had only a small effect on g_t at short circuit and positive V_t, clearly sensitized g_{Cl} at all negative clamp potentials, thus shifting the g–V curve slightly to the right. After CPT-cAMP, g_t was drastically increased at short circuit. Clamping the potential to negative values did not increase g_t markedly, whereas clamping to positive values led to a finite decrease in g_t; it was, however, far less pronounced than under control conditions or with theophylline. Most pronounced are the changes in ion specificity of the g–V relation induced by the application of CPT-cAMP. Under control conditions, NO_3^- and SO_4^{2-} decreased g_t in an almost identical manner, bringing it to similar low values irrespective of voltage. After CPT-cAMP, replacement of apical Cl^- by SO_4^{2-} eliminated the major fraction of the activated conductance, whereas NO_3^- was virtually ineffective. At positive clamp potentials, g_t was then larger with NO_3^- than with Cl^-.

In contrast to the g–V relations under control conditions or with inhibitors of the phosphodiesterase, which always displayed a sigmoidal shape with near-complete inactivation at V_t around +30 mV, response patterns of skins in the presence of CPT-cAMP were qualitatively different and to some degree variable. As shown in Fig. 7, stimulation with CPT-cAMP increased g_t to levels between 1·5 and 6·2 mS cm^{-2} at short circuit; control values of these tissues at short circuit before addition of CPT-cAMP were between 0·38 and 0·82 mS cm^{-2}. In all experiments, clamping to serosal negative potentials did not activate g_t markedly as compared with its value at short circuit; opposite (positive) clamp potentials decreased g_t in one case but were ineffective in six other tissues. It appears from these results that the voltage sensitivity of the Cl^-pathway is mostly neutralized in the presence of CPT-cAMP, and the maximal level of the

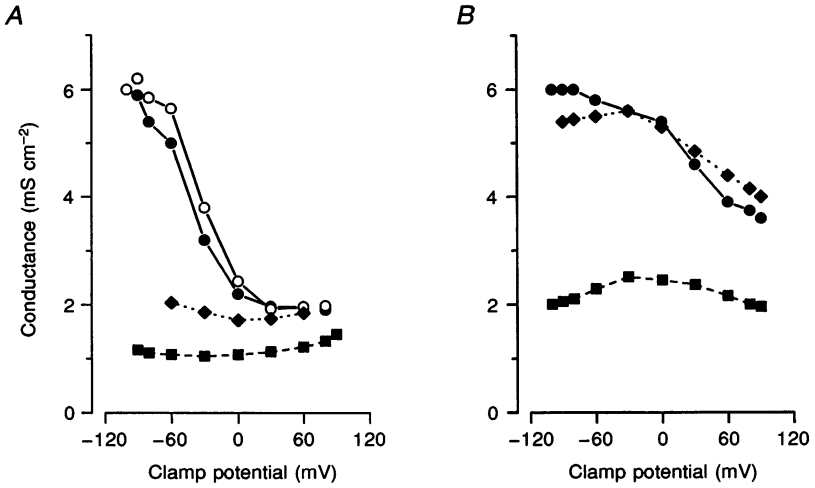

Figure 6. Voltage–conductance relations of a toad skin

A, control conditions with mucosal Cl^- (●), SO_4^{2-} (■) or NO_3^- (◆), and with 1 mM theophylline in Cl^- on the apical side (○). *B*, the same tissue after application of 300 μM CPT-cAMP from the serosal side with mucosal Cl^- (●), SO_4^{2-} (■) or NO_3^- (◆).

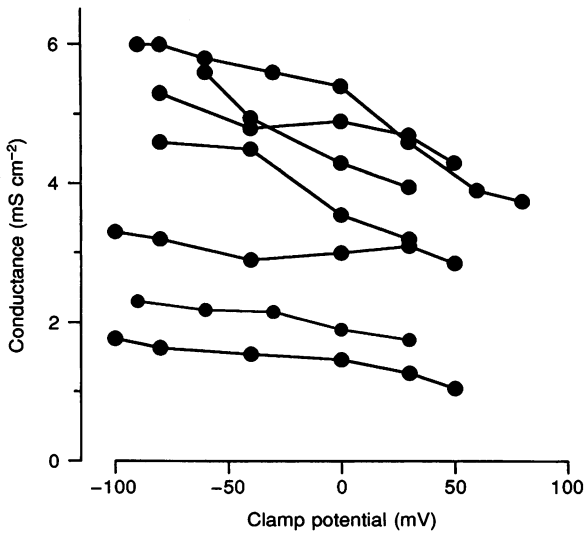

Figure 7
Voltage–conductance relations of 7 separate pieces of toad skin incubated with 300 μM CPT-cAMP and bathed in NaCl Ringer solution on both sides.

g_t achieved is maintained for each particular tissue. It should be noted that in those instances where g_t displayed some change, the final level of conductance was approached nearly instantaneously upon clamping V_t to the new value. This is completely different from the pattern under control conditions or with theophylline, which shows time constants in the order of 1–2 min.

DISCUSSION

Chloride transport across amphibian skin in general and toad skin in particular is a complex process and the underlying mechanism(s) and the morphological route(s) are still unclear. The apical membrane of the stratum granulosum cells is impermeable to Cl^- (Willumsen & Larsen, 1986; Nagel, 1989), and therefore this ion must penetrate the skin at a site separate from the syncytial Na^+ transport compartment. As a possible route for Cl^- transport, MR cells were proposed (Voute & Meier, 1978; Kristensen, 1981). These cells, which account for approximately 2–5% of the apical surface of the skin, are interspersed between the principal cells of the epithelium (reviewed in Larsen, 1991; Katz & Nagel, 1994). Although our experiments do not pertain to the localization of the Cl^--related conductive pathway to any particular intra-epithelial sites, the responsiveness to cAMP suggests that this path involves a cellular compartment. Many epithelial Cl^- channels are sensitive to cAMP (Liedtke, 1989; Reeves & Andreoli, 1992), where it is generally assumed that the phosphorylation–dephosphorylation cycle is responsible for activation of a G-protein that determines the transition from resting to active states and controls the activity of the reaction concerned, i.e. Cl^- conductance in the present case (Heming, Copello & Reuss, 1994). Accordingly, the response of frog and toad skins on supposed changes in cellular cAMP may be considered as evidence for the cellular localization of the Cl^- channels in these tissues, i.e. in the apical membranes of MR cells.

The localization of Cl^- movement to MR cells is still debated. In addition to results from electron probe microanalysis, which demonstrate insufficient gain of a marker ion (Br^-) in MR cells after activation of transepithelial Cl^- current (Dörge, Rick, Beck & Nagel, 1988), recent measurements using a vibrating current probe raised doubts about whether MR cells can account for the associated current flow (Nagel *et al.* 1993). Although peaks of current were localized at the position of MR cells, as observed in previous studies (Foskett & Ussing, 1986; Katz & Scheffey, 1986), the simultaneous determination of total transepithelial clamp current revealed that less than 20% of the applied current could be detected over MR cells.

Our study shows that the baseline Cl^- conductance at short circuit is affected only a little by low concentrations of cAMP and its analogues, whereas it is strongly elevated by higher concentrations of the membrane-permeable, non-metabolized analogues of cAMP. At the same time, the voltage-activated gain in g_{Cl} is reduced at low concentrations and completely eliminated at higher concentrations of cAMP. These effects are entirely reversible and similar to those of forskolin (Nagel & Van Driessche, 1992). Theophylline and IBMX, on the other hand, exert qualitatively different effects. These drugs have a smaller effect as regards the baseline conductance, but they sensitize the voltage-activated Cl^- conductance, unless the voltage sensitivity of g_{Cl} is already high under control conditions. Although both phosphodiesterase inhibitors have been demonstrated to increase the intracellular level of cAMP considerably in concentrations used in the present and previous studies (Johnsen & Nielsen, 1978), and despite the fact that the relation between maximally effective concentrations of theophylline and IBMX for stimulation of g_{Cl} corresponds to the inhibitory power of the agents on phosphodiesterase preparations (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970), it is difficult to predict how intracellular concentrations of cAMP after inhibition of the phosphodiesterase compare with those effectively built up with the cAMP analogues. The fundamentally different response patterns suggest, on the

other hand, that the effect on g_{Cl} exerted by theophylline or IBMX is not, or only secondarily, due to the increased cellular cAMP. Since rather high concentrations of cAMP are evidently required to raise the baseline g_t, levels of cAMP achieved after application of the inhibitors might not be effective enough to activate the voltage-sensitive site in the permeation pathway into a permanently open state. These concentrations may, on the other hand, be sufficient to sensitize the activation by electrical voltage, as was suggested by Willumsen *et al.* (1992), from the shift of the $g-V$ relation to the right. It may be pertinent in this context to mention that baseline g_{Cl} in frog skin, which can be activated by theophylline (Kristensen, 1970), shows similar responsiveness to apical addition of procaine (Flonta, Endsrasser, Kiermeyer & Nagel, 1988). The latter compound and several other local anaesthetics appear to act by virtue of and in proportion to their lipophilic nature (Nagel, 1989). Theophylline and IBMX are also lipophilic and could act in a similar way rather than by the inhibitory effect on the phosphodiesterase. This could explain the observation that application of theophylline after maximal stimulation with CPT-cAMP partly restored voltage sensitivity.

A number of observations warrant consideration in this context. (1) Maximal magnitudes of the voltage-activated g_{Cl} with or without theophylline and the cAMP-induced Cl^- conductance at short circuit are often different, the latter being generally larger. It was reported recently (Willumsen *et al.* 1992) that maximal conductance was similar upon voltage activation both in the absence and presence of cAMP. One of the differences between the present and the previous study is that we used CPT-cAMP which we find to be considerably more potent than dB-cAMP. Furthermore, split skin as used by Willumsen *et al.* (1992) is less sensitive to stimulation of g_{Cl} than intact skin. (2) Voltage sensitivity of the cAMP-induced g_t does not compare with the voltage activation under control conditions. The difference in time course is particularly evident. Contrary to the time-dependent, slow exponential rise of g_{Cl} in the absence of cAMP, this component was removed upon its application, and voltage perturbation affected the conductance almost instantaneously (see Fig. 3). Cyclic AMP, even at low concentrations, seems to transform the voltage-activated site into a different state that has lost the voltage sensitivity, whereas time-dependent voltage activation of conductance remains essentially the same after theophylline and IBMX. It corroborates the view that the effect of these agents is not due only to the elevation of cellular cAMP. The maximal increase in the conductance induced by cAMP analogues was usually already obtained at short circuit and g_t often even decreased after voltage perturbations of either polarity. These patterns could be explained if the conductance of the Cl^- pathway was not determined only by the rate limiting entry step. Consecutive passage through finite compartments could establish an additional rate limitation, perhaps due to ion depletion, for example. (3) The fluctuation in voltage-induced Cl^- current noise has been associated with randomly opening conductance pathways (Nagel & Van Driessche, 1991). After CPT-cAMP, the magnitude of the plateau value of these fluctuations was decreased to less than 50% although the overall current had increased. This observation agrees with previous findings after elevation of cellular cAMP with forskolin (Nagel & Van Driessche, 1992). It may be speculated that this behaviour results from a decrease in fluctuating units after cAMP that have lost the intrinsic gating property with open probability approaching unity. Thus they do not show up in the noise spectrum.

The Cl^- pathway across amphibian skin epithelium appears to be composed of two basic elements (Larsen & Rasmussen, 1983): (1) an anion-specific channel with relatively weak selectivity among halides (Harck & Larsen, 1986), and (2) a voltage-sensitive modifier site, which determines the access of permeable anions to the Cl^- conductive path. These two components must be considered in any model of the regulation of transepithelial Cl^- conductance. Evidently, the permeability of the channel to individual anions may be different from the effectiveness of the respective anions on the voltage-activated control sensor (Harck & Larsen, 1986), and some anions, such as NO_3^-, SCN^- and I^-, even inhibit opening of the gate. Our observations suggest that high concentrations of cAMP transform the voltage sensitivity of the gate into a new state that is voltage insensitive and is maximally opened. Yet, the strongly blocking anions SCN^- and I^- are still able to obliterate the path, whereas the less potent inhibitor, NO_3^-, becomes essentially ineffective at the sensor and can penetrate the anion channel. Thus, cAMP seems to affect the two components of the same pathway, presumably independently of each other.

In conclusion, our experiments show that theophylline and cAMP exert different effects on g_{Cl} (baseline, at short circuit and voltage activated) in toad skin, which cannot be explained by elevation of cellular cAMP alone. The chloride-conductive pathway in toad skin can be divided into separate functional elements, i.e. an anion-specific conducting path and a voltage-sensitive control element. The interaction and biochemical basis of these components, and their intimate control remain to be established.

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